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A Photo-Crosslinked Poly(vinyl Alcohol) Hydrogel Growth Factor Release Vehicle for Wound Healing Applications

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ABSTRACT

The objective of this study was to develop and evaluate a hydrogel vehicle for sustained release of growth factors for wound healing applications. Hydrogels were fabricated using ultraviolet photo-crosslinking of acrylamide-functionalized nondegradable poly(vinyl alcohol) (PVA). Protein permeability was initially assessed using trypsin inhibitor (TI), a 21 000 MW model protein drug. TI permeability was altered by changing the solids content of the gel and by adding hydrophilic PVA fillers. As the PVA content increased from 10% to 20%, protein flux decreased, with no TI permeating through 20% PVA hydrogels. Further increase in model drug release was achieved by incorporating hydrophilic PVA fillers into the hydrogel. As filler molecular weight increased, TI flux increased. The mechanism for this is most likely an alteration in protein/gel interactions and transient variations in water content. The percent protein released was also altered by varying protein loading concentration. Release studies conducted using growth factor in vehicles with hydrophilic filler showed sustained release of platelet-derived growth factor (PDGF-β,β) for up to 3 days compared with less than 24 hours in the controls. In vitro bioactivity was demonstrated by doubling of normal human dermal fibroblast numbers when exposed to growth factor–loaded vehicle compared to control. The release vehicle developed in this study uses a rapid and simple fabrication method, and protein release can be tailored by modifying solid content, incorporating biocompatible hydrophilic fillers, and varying protein loading concentration.

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INTRODUCTION

The promise of dramatic increases in wound healing rate and quality using growth factors such as platelet-derived growth factor (PDGF) has to date not been realized. Many clinical studies have failed to show effectiveness of bolus administration of single growth factors, and Regranex gel, the only commercially available wound treatment using PDGF, shows only a moderate increase in the incidence of complete healing of diabetic ulcers (from 25% and 35% in placebo groups to 48% and 50% in treated groups, respectively[1,2]). As described by Margolis et al,[3] the effect of PDGF on healing wounds is moderate probably because of inefficient delivery to the cells needed to heal the wound. When growth factors are administered as a bolus, rapid clearance from the wound site occurs, making it difficult to maintain therapeutic concentration over prolonged periods of time.

Sustained release of PDGF has been explored for regeneration of bone,[4-7] periodontal bone,[8-11] and tympanic membrane,[12] and formation of mature vascular networks.[13] These studies used vehicles such as the bioactive ceramic hydroxyapatite, ethylene vinyl acetate, chitosan and poly-l-lactide. In this article, a novel poly(vinyl alcohol) (PVA) hydrogel-based vehicle for sustained release of growth factors in chronic wounds was designed. The processability, strength, and long-term temperature and pH stability of PVA hydrogels, together with their low toxicity and minimal cell and protein adhesion,[14] make them ideal candidates for the design of a sustained release vehicle.

Numerous reports have described the use of hydrogels in controlled release formulations.[15,16] PVA hydrogels are versatile hydrophilic polymer networks whose properties

Table 1. Hydrogel Formulations Used for Characterization*

Label	Composition		Total Solid Content at Fabrication (%)
	Matrix: a-f PVA 83% Hydrolyzed†	Filler: nf PVA 99% Hydrolyzed‡	
10	10% a-f PVA	None	10
15	15% a-f PVA	None	15
20	20% a-f PVA	None	20
2.5 LMW	15% a-f PVA	2.5% nf PVA (LMW)	17.5
5 LMW	15% a-f PVA	5% nf PVA (LMW)	20
2.5 MMW	15% a-f PVA	2.5% nf PVA (MMW)	17.5
5 MMW	15% a-f PVA	5% nf PVA (MMW)	20
2.5 HMW	15% a-f PVA	2.5% nf PVA (HMW)	17.5
5 HMW	15% a-f PVA	5% nf PVA (HMW)	20

*a-f indicates acrylamide-functionalized; HMW, high molecular weight; LMW, low molecular weight; MMW, medium molecular weight; MW, molecular weight; nf, nonfunctionalized; PVA, poly(vinyl alcohol).
†MW = 14 000.
‡MW: 13 000 to 23 000 (LMW), 31 000 to 50 000 (MMW), or 124 000 to 186 000 (HMW).

can be controlled by variation of the degree of residual acetylation (percent hydrolysis) as well as by variation of the crosslink density.[17] PVA-based hydrogels suitable for application to wounds can be obtained by chemical or physical crosslinking.[18] Unfortunately, most of the existing methods used for crosslinking involve steps that can have adverse effects on protein drugs. These include crosslinking of proteins via glutaraldehyde treatment, protein denaturation and aggregation during freeze-thaw treatment, and radiation-induced crosslinking or chain scission of loaded proteins. In this study we evaluated the potential of a novel UV photo-crosslinked PVA hydrogel[19] as vehicle for controlled delivery of protein drugs. This PVA hydrogel is crosslinked via free radical formation acting on acrylamide functional pendant groups. This fast crosslinking technology allows minimal exposure to ultraviolet radiation and does not require addition of chemicals or other treatments to the hydrogel.

A typical problem with delivery of low molecular weight proteins from hydrogels is an initial "burst" and lack of control during the release.[20] The principle of adding fillers and excipients to drug release vehicles is commonly used to enhance protein stability and shelf life as well as to allow for ease of formulation and fine control of release profiles.[21-23] Hydrophilic PVA fillers were selected in this study, as they are compatible with the hydrogel matrix and can be supplied across a wide range of molecular weights.

The primary aim of this study was to characterize a novel UV photo-crosslinkable PVA hydrogel by determining the effects of solid content, incorporation of hydrophilic PVA fillers, and initial protein loading concentration on protein permeability and release of the model drug, trypsin inhibitor. The second goal was to formulate a growth factor delivery vehicle to determine the release profile of PDGF-β,β and to evaluate the bioactivity of released PDGF-β,β on normal human dermal fibroblasts.

MATERIALS AND METHODS

Hydrogel Formulations and Preparation

A series of formulations (**Table 1**) were investigated to understand the effects of variations in matrix solid content and use of fillers on the parameters of swelling equilibrium, partition coefficient and permeability. Additionally, the effect of protein loading concentration on protein release profile of hydrogels with different solid contents was examined.

Fillers

Nonfunctionalized PVAs of low (13 000-23 000), medium (31 000-50 000), and high (124 000-186 000) molecular weight (referred to as LMW, MMW, and HMW, respectively) in pellet form were purchased (PN34840, PN36313-8, and PN 36316-2, respectively, Sigma, Inc, St Louis, MO; 99% hydrolysis) and used as filler. Filler stock solutions (15% wt/vol) were prepared by dissolving pellets in ultrapure water (resistivity of 16-18 MΩ-cm) obtained from a nanopure system (Barnstead/Thermolyne, Dubuque, IA) by stirring overnight at 65°C. Final concentrations of fillers in 15% solid content hydrogel matrix were 0, 2.5 or 5% (wt/vol).

Proteins

The model drug used for partition coefficient, permeability, and release studies was soybean trypsin inhibitor (TI) (21 000 MW, Sigma). TI was chosen because of its molecular weight and structural similarities to PDGF. A 75 mg/mL protein stock solution in nanopure water was prepared and stored at –20°C until needed. PDGF-β,β powder was purchased from Chemicon Intl (Temecula, CA). The growth factor was reconstituted to 100 µg/mL in 10mM acetic acid and stored frozen at –20°C in 10 µL aliquots.

Hydrogel Matrix

Poly(vinyl alcohol) (PVA) hydrogels containing 10%, 15% or 20% solid content were fabricated from a 30% stock solution of an acrylamide-functionalized PVA with 14, 000 average molecular weight (Mowiol 3-83; 83% hydrolyzed; acrylamide-functionalized variant supplied by BioCure, Inc, Atlanta, GA) containing 0.1% Irgacure2959 as a photo initiator.[19]

Matrix hydrogel, filler and water were first combined and slowly vortexed overnight at room temperature to ensure that all solutions were homogeneous. Thereafter, solutions were filtered using a 0.45 µm syringe filter. For release and bioactivity studies, the selected protein was added and mixed to achieve homogeneity. Hydrogels were fabricated from the solutions by UV crosslinking. The appropriately formulated solutions were pipetted into molds consisting of a rubber washer (thickness = 1.51 mm, diameter = 15.9 mm) placed on top of a glass microscope slide, covered with coverslips and UV irradiated for 60 seconds using a Green Spot UV (UV Source, Torrance, CA) (peaks at 310 and 365 nm, 2 mW/cm^2). For permeability and partition coefficient studies, no protein was added to the hydrogels and they were used as obtained from the mold. For all other experiments, samples of 5 mm diameter were punched out of the hydrogel with a biopsy punch. For calculation of total protein loaded, hydrogel volumes were calculated from their dimensions based on mold dimensions and biopsy punch diameter.

Characterization of Hydrogels

Swelling Equilibrium (q)

Immediately after fabrication, the hydrogels (n = 4) with and without filler were weighed and transferred into phosphate-buffered saline (PBS) (10 mM, pH 7.4; Sigma P-3813) and incubated at 37°C. Hydrogels were removed from PBS, gently blotted dry, and reweighed at 1, 2, 4, 24, 48, and 72 hours when they reached constant weight. At the completion of the water uptake, the hydrogels were weighed (m_{tot}), followed by drying to constant weight (m_p, mass of polymer). Swelling equilibrium (mass) (q) was calculated as follows:

$$q = m_{tot}/m_p \quad (1)$$

Partition Coefficient

To determine the partition coefficient, individual hydrogels were equilibrated in PBS overnight followed by incubation in 2 mL of a 1 mg/mL trypsin inhibitor in PBS at 37°C. After 3 days, the amount of trypsin inhibitor remaining in the solution was measured by high pressure liquid chromatography (HPLC) as described below. Protein concentrations were determined by a calibration curve. The partition coefficient, K_d, was calculated as follows[14]:

$$K_d = \frac{C_m}{C_s} = \frac{V_s(C_0 - C_e)}{V_m C_0} \quad (2)$$

where K_d is the ratio of the trypsin inhibitor concentration in the membrane, c_m, to the trypsin inhibitor concentration in the solution at equilibrium, c_s. V_s and V_m are the volumes of trypsin inhibitor in solution and hydrogel, respectively; c_0 is the initial concentration of trypsin inhibitor in solution, and c_e is the trypsin inhibitor concentration in the solution at equilibrium.

Permeability

Permeability studies were conducted using vertical Franz diffusion cells. The apparatus consisted of a receptor and a donor compartment. At the time of the experiment, the receptor compartments of vertical Franz diffusion cells (diameter = 5 mm, and volume = 4.1 mL) were filled with PBS and stirred at 600 rpm (37°C). After equilibration in PBS, hydrogels (n = 3) with or without filler were placed on top of the receptor compartments. The donor cells were then placed on top of the hydrogels. Exactly 250 µL of TI solution (10 mg/mL) in PBS was placed in the donor compartments and occluded with Parafilm to prevent evaporation. Samples (300 µL) were collected from the receptor compartments over 5 days (2, 4, 8, 12, 24, 36, 48, 60, 72, 84, 96, 108, and 120 hours). The withdrawn volumes were immediately replaced with PBS. Samples were kept frozen until analysis by HPLC. Permeability was plotted as cumulative amount of TI measured in the receptor cell (µg/cm^2)

versus time. Flux (μg/cm^2-h) was calculated from the slope of the linear segment of the permeability curve.

Protein Release Studies

Hydrogels of 10%, 15%, and 20% solid content matrix with final TI concentrations of 0 (controls), 5, 10 or 20 mg/mL hydrogel were fabricated as described above. Immediately after fabrication, samples were individually incubated in 1.5 mL of PBS sink. Sample vials were kept under constant stirring in a water bath shaker (Orbital Water Shaker 3540, LabLine, Melrose Park, IL) at 37°C. Aliquots (500 μL) were removed followed by equal volume replacement with PBS at regular intervals up to 7 days. Samples were stored frozen at –20°C prior to analysis by HPLC as described below.

Analysis of Trypsin Inhibitor Concentration by HPLC

TI concentration for partition coefficient, permeability, and release studies samples was analyzed using an hp1090 HPLC system (Hewlett-Packard, Palo Alto, CA) comprising a quaternary pump (model 79852A), an autosampler (model 79855A), and a variable wavelength UV detector (model 79853C). The concentration of TI in samples was calculated from a calibration curve using external standards of known concentrations of trypsin inhibitor at a detection wavelength of 280 nm. The potential for interference with absorbance by PVA (or components of the release system) was assessed by running UV scans (190-300 nm) on solutions of PVA and trypsin inhibitor. No interference was observed at the concentrations of TI and PVA likely to be in solution. In addition, control hydrogels without trypsin inhibitor were included in the release study and no peaks were detected on the chromatogram. The column used for separations was a Phenomenex Jupiter 5m C18, 30 nm, 150 × 2 mm with Phenomenex SecurityGuard universal guard cartridge system (Phenomenex, Torrence, CA). The mobile phase used was based on a gradient elution of phases A (0.1% trifluoroacetic acid [Acros Organics,Morris Plain, NJ] in water [HPLC grade; Pharmco, Brookfield, CT]) and B (0.1% trifluoroacetic acid [Acros] in acetonitrile [HPLC grade; Pharmco]). Initial mobile phase started at 90% A and 10% B, reaching 10% A and 90% B by 15 minutes, and returning to initial mobile phase by 16 minutes and equilibrated up to 20 minutes. Injection volume and flow rate were 40 μL and 0.4 mL/min, respectively.

Effectiveness of Filler Entrapment in Hydrogel Matrix

These experiments were performed to assess the extent of filler released from the hydrogel matrix during incubation. The dry weight of the 5 mm diameter hydrogels was determined by thermogravimetric analysis (TGA: Hi-Res TGA 2950 Thermogravimetric Analyzer, TA Instruments, New Castle, DE) at time of fabrication, and after incubation in PBS for 5 and 7 days. Two hydrogels per composition per time point were fabricated. Samples (n = 4) were taken from the hydrogels by using a biopsy punch and run at 10°C/min from 25°C to 200°C. Results are plotted as hydrogel dry weight before and after incubation in PBS.

Release Vehicle for PDGF

Formulation

A 15% solid content PVA hydrogel matrix with 5% filler (HMW: nonfunctionalized HMW PVA, 99% hydrolysis) was selected as the release vehicle (RV) formulation for the delivery of PDGF-β,β. HMW-filled RV was pursued further primarily because in these hydrogels the filler was retained for up to 5 days (as compared with LMW and MMW fillers, which appeared to be lost by 5 days) and because increase in MW of fillers appeared to cause dramatic increases in TI flux. A 15% solid content PVA hydrogel matrix without filler was used as control. Hydrogels were fabricated as described above under aseptic conditions. PDGF-β,β was added at a final concentration of 0.8 μg PDGF-β,β/mL hydrogel solution. Individual hydrogels contained a total loading of 23.6 ng PDGF-β,β. Both the release of PDGF-β,β and its bioactivity on normal human dermal fibroblasts were assessed by 2 independent experiments, as described below.

PDGF-β,β release

Release studies were conducted immediately after hydrogel fabrication. Release vehicles and controls (n = 4/group) were individually placed in 500 μL PBS sink. Sample vials were under constant stirring at 37°C (Orbital Water Shaker 3540, LabLine, Melrose Park, IL). Total sample removal followed by equal volume fluid replacement was done at 4, 8 and 16 hours, and 1, 2, 3, 5 and 7 days. PDGF-β,β release was determined by a quantitative enzyme-linked immunosorbent assay according to manufacturer's instructions (Quantikine, Human PDGF-β,β Immunoassay, Catalog No DBB00, R&D Systems, Inc, Minneapolis, MN) using the protocol provided by the manufacturer of the assay.

Table 2. Test Groups for Bioactivity Assay*

Formulation Name	Filler Content	PDGF-β,β Content
Control	None	None
Control with supplemented PDGF-β,β	None	Bolus 20 ng/mL
Control with loaded PDGF-β,β	None	23.6 ng/gel
RV	5% HMW	None
RV with supplemented PDGF-β,β	5% HMW	Bolus 20 ng/
RV with loaded PDGF- β, β	5% HMW	23.6 ng/gel

*HMW indicates high molecular weight; PDGF, platelet-derived growth factor; RV, release vehicle.

Table 3. Mass Swelling at Equilibrium (*q*), Partition Coefficients (K), and Protein Flux (μg/(cm^2.h)) of PVA Hydrogels With and Without Fillers*

Hydrogel Formulation		Swelling Equilibrium, *q*	Partition Coefficient, K	Flux μg/(cm^2 x h)
Matrix (%)	Filler (%)			
10		6.0 ± 0.2	6.6 ± 0.0	18.0 ± 2.2
15		4.9 ± 0.4	2.5 ± 0.1	1.9 ± 0.8
20		3.8 ± 0.2	1.9 ± 0.5	0
15	2.5 LMW	5.0 ± 0.4	ND	ND
15	5 LMW	4.6 ± 0.2	1.5 ± 0.4	3.0 ± 1.1
15	2.5 MMW	5.0 ± 0.1	ND	ND
15	5 MMW	5.0 ± 0.3	1.9 ± 0.2	5.9 ± 3.7
15	2.5 HMW	4.4 ± 0.3	ND	ND
15	5 HMW	4.6 ± 0.7	1.3 ± 0.6	86.8†

*Values reported are mean ± standard deviation. HMW indicates high molecular weight; LMW, low molecular weight; MMW, medium molecular weight; ND, not determined.
†n = 1. Mechanical properties of these hydrogels were stiffer than others, and it was difficult to mount them in Franz cells without fracture.

Bioactivity of Released PDGF-β,β Using Normal Human Dermal Fibroblasts

The effect of released PDGF-β,β from RV and controls on normal human dermal fibroblast (NHDF) proliferation was compared to that of supplemented PDGF-β,β bolus in the medium. Usually, tissue culture medium is supplemented with fetal bovine serum (FBS) to supply cells with appropriate nutrients and factors necessary for normal cell growth and function. Complete medium with 10% to 15% FBS contains concentrations of growth factors that could mask the effects of PDGF-β,β released by the RV. Preliminary experiments (not reported here) determined that optimal FBS concentrations necessary to maintain normal cell growth and still observe the effects of added PDGF-β,β was of the order of 6% FBS.

The control and experimental groups (n = 4 hydrogels/time point) are described in **Table 2**. NHDFs (Clonetics Biowhittaker Corp, Walkersville, MD) were allowed to reach confluence in complete medium (fibroblast growth medium-2 containing 2% FBS, human fibroblast growth factor-B [hFGF-B] [1 mg/mL], insulin [5 mg/mL], and gentamicin [30 mg/mL]–amphotericin [15 μg/mL] [GA-100]; Clonetics Biowhittaker). Cells were seeded at a density of 3200 cells/well in a 96-well tissue culture polystyrene plate (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) and allowed to attach overnight (18-20 hours) in complete medium. Cells were then starved for 48 hours by replacing complete medium with fibroblast basal medium (FBM) (Clonetics Biowhittaker) containing 0.5% FBS. To initiate the bioactivity assay, the medium was carefully removed and hydrogels were gently placed on the cell layer with sterile forceps without disturbing the cell layer. FBM con-

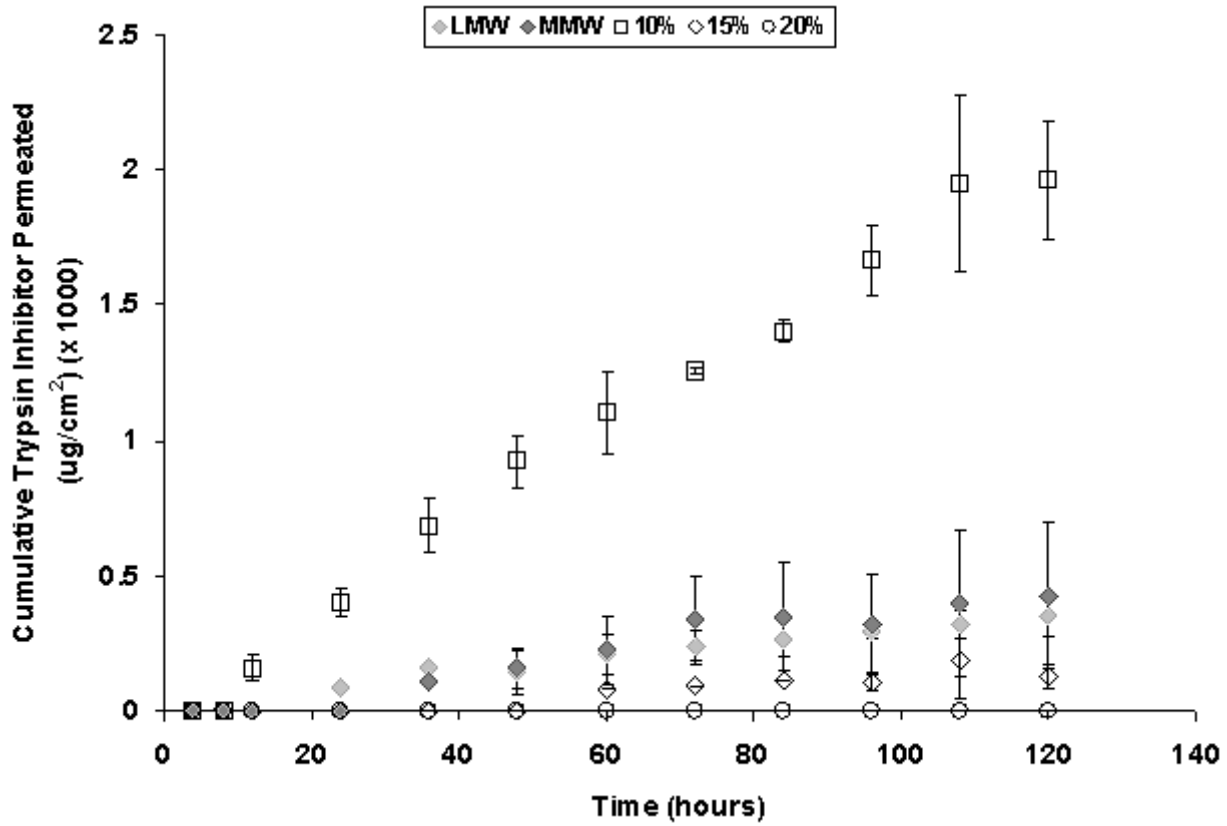

Figure 1. Effect of solid content and incorporation of 5% LMW, MMW, and HMW fillers in 15% hydrogel matrix on the permeability of trypsin inhibitor. No TI permeation was observed in the 20% hydrogel over the experimental period. Error bars represent standard deviation (n = 3) (HMW: n = 1).

taining 6% FBS (with or without supplemented PDGF-β,β [20 ng/mL]) was added (day 0). Cells were harvested at 0, 1, 3, 5, or 7 days and the medium was changed on days 2, 4, and 6 for remaining plates (FBM containing 6% FBS; with or without supplemented PDGF-β,β [20 ng/mL]). Cell number was estimated using the metabolic colorimetric MTS assay according to the manufacturer's instructions (CellTiter 96 Aqueous One Solution Reagent, Promega cat#:3580; Promega Madison, WI).

RESULTS

Characterization of Hydrogels

Swelling Equilibrium

The equilibrium degree of swelling (q) is a reflection of the hydrophilicity of the matrix. **Table 3** shows values obtained for q for all hydrogel formulations. For the PVA hydrogel matrix alone, q increased (from 3.8 to 6.0) with decreasing solid content. The incorporation of LMW, MMW, or HMW fillers at concentrations of 2.5% or 5% into a 15% hydrogel matrix increased the effective solid content to 17.5% or 20%, respectively (**Table 1**). However, the mass swelling at equilibrium in these filled hydrogels remained equivalent to that of 15% hydrogel matrix. This indicates either increased water uptake or the loss of filler over the incubation period of 3 days. In the hydrogels filled with 2.5% HMW filler, q was slightly lower, closer to that measured for 20% hydrogels. Interestingly, the results showed an initial increase in water uptake in 15% hydrogels with HMW filler resulting in a peak at approximately 1 hour followed by a continuous decrease in weight until reaching equilibrium between 48 and 72 hours (data not shown). Similar peaks were not observed in hydrogels without filler or with LMW and MMW filler. This observation indicates that the HMW filler had major effects on the water uptake in the initial incubation period that may not be reflected in the equilibrium water content.

Partition Coefficient

Partition coefficient is a measure of the partitioning of the solute (TI) between the gel and the surrounding solution. If a solute has high affinity for the gel as a result of electrostatic, hydrophobic interactions or other interactions, K >> 1, whereas if the solute prefers the external solution or is excluded on the basis of size, K < 1. Partition coefficients of 1 indicate no preference. The results shown in **Table 3** suggest that as the solid content increases the preference of TI for the gel decreases or it is excluded from the gel. In the case of PVA hydrogels, charge is probably not an important determinant of K, whereas "mesh" size and hydrophobic interactions may play a significant role. When 5% filler was incorporated into the 15% PVA matrix, partition coefficients decreased and were equivalent to those of a 20% solid content matrix, independent of filler molecular weight. The result is paradoxical since the swelling behavior of filled hydrogels suggested that these hydrogels behave like 15% unfilled hydrogels.

Permeability

These studies were conducted to characterize the permeability of TI in different hydrogels tested in the absence of the confounding effects of protein entrapment within hydrogels. The cumulative amount of trypsin inhibitor permeated through the hydrogels up to 5 days is shown in **Figure 1** and protein flux for each hydrogel variant is given in **Table 3**. As expected, increasing the solids content of unfilled hydrogels decreased the flux of TI. Flux of the 21 000 MW model drug was approximately 10 times higher in 10% unfilled hydrogels compared with 15% hydrogels. No TI permeated through the 20% hydrogels. The higher permeability of TI in 10% hydrogels may be associated with their higher degree of swelling (**Table 3**), which increases the available free volume for diffusion and the greater "preference" of TI for these 10% hydrogels (indicated by K_d), allowing protein to move into the gel and down the concentration gradient.

Incorporation of LMW, MMW, and HMW PVA fillers into the 15% PVA hydrogel (at 5% concentration and 99% hydrolysis) resulted in significantly increased permeability of TI through the hydrogels (**Figure 1**, **Table 3**). Incorporation of fillers increased flux of TI in 15% hydrogels by approximately 1.5 times (LMW filler) and 3 times (MMW filler) compared to 15% hydrogel without filler. Data for the hydrogel with HMW hydrophilic filler were difficult to obtain because of mechanical properties of the gel. However, in one successful experiment, a greater than 40-fold increase in TI permeability was noted. When the mass swelling is examined (**Table 3**) it is clear that there is a consistent (expected) decrease in *q* from 10% up to 20% PVA, the 10% having lower solid content and higher free volume available for diffusion. However, decrease in swelling was not associated with a consistent decrease in flux; in fact, there appears to be a critical solids content for flux of TI between 15% and 20% PVA.

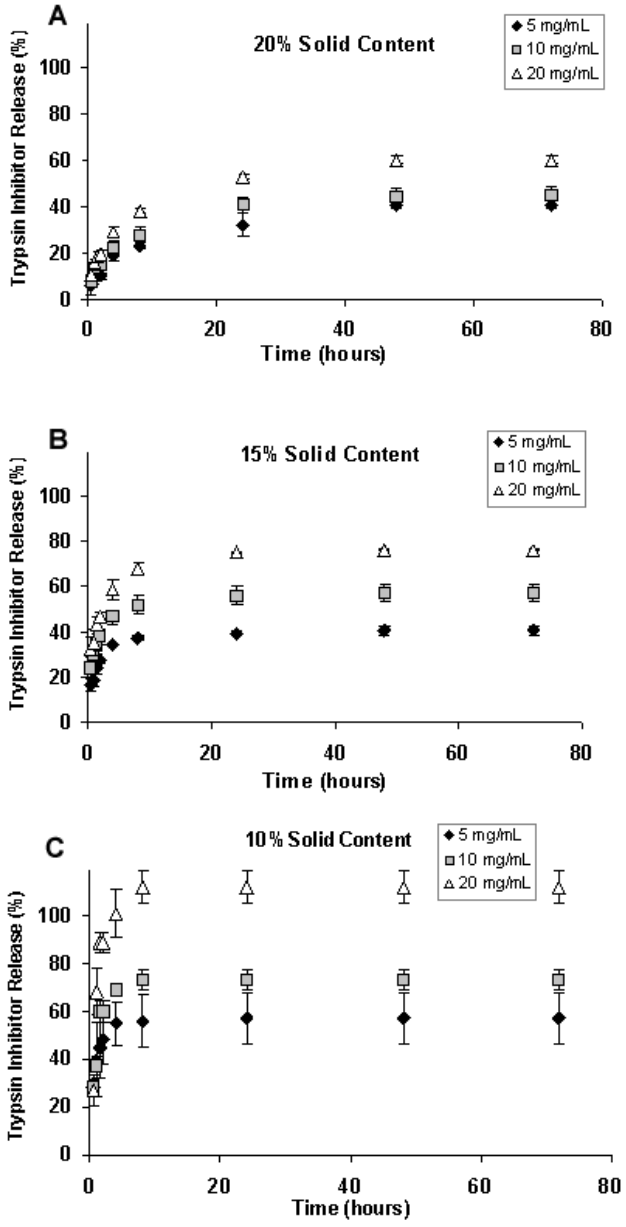

Figure 2. Effect of protein loading concentration (5, 10, and 20 mg/mL) on the protein release from (A) 20% PVA, (B) 15% PVA, and (C) 10% PVA content hydrogel matrix. Error bars represent standard deviation (n = 4).

Protein Release Following Entrapment

Results for TI release from hydrogels of 15% to 20% PVA loaded with between 5 and 20 mg/mL are shown in **Figure 2**. Note that quantitation of the initial content of TI in the hydrogel was based on protein concentration in stock solution and calculation of the hydrogel volume from its dimensions. The hydrogel diameter was 5 mm and the thickness was 1.51 mm (based on mold dimensions). Actual hydrogel dimensions depend on the fabrication accuracy and biopsy punch accuracy, and a slight deviation in dimensions can lead to an error. For example, with variability of radius and thickness of up to 0.1 mm, the total variability in percent release could be as high as 10% to 15%, which could account for the values higher than 100% in **Figure 2C**. Corresponding with the permeability data, the rate of protein release from 20% hydrogels was slower compared with that from 10% PVA hydrogels. Regardless of entrapped protein concentration, maximal release occurred between 24 and 48 hours in 20% PVA hydrogels (**Figure 2A**). In 15% PVA hydrogels, maximal release occurred before 24 hours and in 10% hydrogels by approximately 8 hours. **Figure 2** also illustrates that the percentage of total entrapped protein released significantly increased in 10% PVA hydrogels compared with 15% and 20% PVA hydrogels. Protein loading concentrations also increased release because when higher loading concentrations were used, higher percent release occurred. The most pronounced effect was observed in the 10% solid content hydrogel, where the percent of loaded protein released increased from 57% to approximately 100% when protein loading concentration was increased from 5 to 20 mg/mL. Accordingly, for 20% and 15% solid content the percent protein released rose from 41% and 42% to 60% and 76%, respectively. Thus, percent protein release was affected by varying loading concentration of the vehicle, and both percent release and the rate of release were perturbed by altering the solid content of the PVA matrix.

Effectiveness of Filler Entrapment in Hydrogel Matrix

As shown in **Figure 3**, all filled hydrogels had dry weights at time zero corresponding to their solids content of 20% (15% matrix + 5% filler). However, following incubation in PBS for 7 days, loss of weight was observed, resulting in reduction of the dry weight to that equivalent to the 15% unfilled hydrogels. This suggests that filler was released from the gel and that complete filler release appeared to occur by 5 days in LMW and MMW fillers and by 7 days in HMW filled hydrogels. Unfilled hydrogels showed no weight loss over 7 days' incubation in PBS. These results suggest that although loss of filler appeared to occur, this was slower for hydrogels with HMW filler. The lack of weight loss in the unfilled hydrogel confirmed that there was no degradation of the matrix over 7 days in vitro.

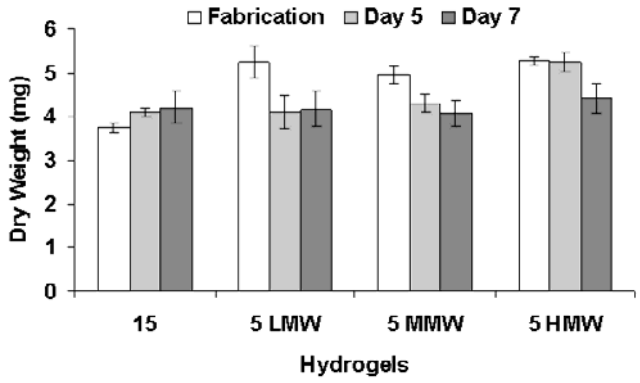

Figure 3. Effectiveness of filler entrapment in hydrogel matrix presented as dry weight (mg) monitored by TGA. Error bars represent standard deviation (n=4).

Release Vehicle for PDGF

PDGF-β,β Release

Figure 4 shows the cumulative release of PDGF-β,β from RV with filler compared with the control. Maximal release occurred by 24 hours in the control, similar to results for TI release from 15% PVA, whereas release reached a plateau at approximately 3 days in the RV with filler. The total amounts of PDGF-β,β released were of the order of 2 times greater from the RV (1.47 ± 0.07 and 3.11 ± 0.56 ng for control and release vehicle, respectively). Given that the amount of PDGF loaded was 23.6 ng per hydrogel, the percent released was low, confirming the earlier finding that loading concentration is a significant factor in determining percent release.

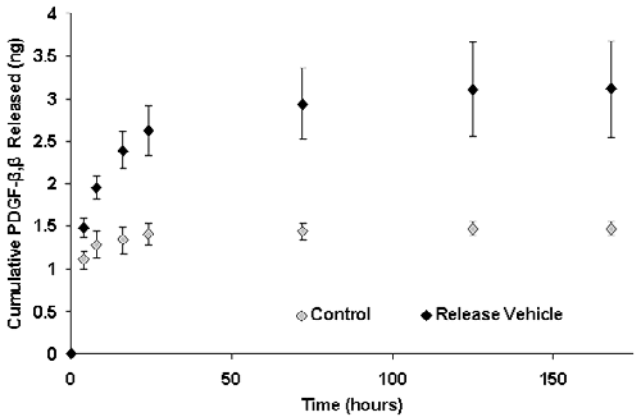

Figure 4. Cumulative release of PDGF-β,β from release vehicle and control. Error bars represent standard deviation (n = 4).

Bioactivity of Released PDGF-β,β Using Normal Human Dermal Fibroblasts

To determine the bioactivity of released PDGF-β,β from RV and control vehicles, NHDFs were cultured under low serum conditions to reduce proliferation. **Figure 5** illustrates the growth curves of NHDF exposed to control (unfilled) hydrogels and to filled RV with and without incorporated PDGF-β,β and supplemented PDGF-β,β (bolus in the medium). By day 5 (**Figure 6**), it was apparent that there was only a slight difference in growth between control hydrogels and control hydrogels loaded with PDGF-β,β. However, when PDGF-β,β was loaded into the RV, NHDF growth was almost double that observed in the control. Adding PDGF-β,β as a bolus supplement in the medium also significantly increased the NHDF growth over control and RV without PDGF-β,β. However, it is important to point out that the total dose by 5 days of PDGF-β,β released from control and vehicle (1.47 and 3.11 ng, respectively, according to release studies) was significantly lower than that of the supplemented bolus (60 ng).

DISCUSSION

In this study, a hydrogel-based system for improving delivery of growth factors to chronic cutaneous wounds was designed. The vehicle was based on a novel photocrosslinkable PVA hydrogel matrix with a PVA hydrophilic filler incorporated, and the specific growth factor used was PDGF-β,β. The results demonstrate that UV-crosslinked PVA can be used for release of LMW proteins and peptide growth factors of the order of 20 000 MW. Additionally, protein permeability of these hydrogels can be tailored by modifying the solid content of the matrix and by incorporating hydrophilic fillers.

The mechanism by which hydrophilic fillers incorporated into the PVA matrix perturb protein permeability is unclear. Permeability studies showed a dramatic increase in flux of the model protein TI when hydrophilic filler was incorporated in the matrix. However, equilibrium swelling (q) did not appear different in filled hydrogels (effective solids of 17.5% or 20%) compared with 15% PVA unfilled hydrogels. This finding coupled with the dry weight data (**Figure 3**) suggests that filler was largely released after 5 days in LMW- and MMW-filled hydrogels. Paradoxically, the partition coefficients suggest that even though filler release occurs, protein partitions in these hydrogels as if total complement of filler was present (**Table 3**). It appears from these results that although in terms of swelling behavior the filled hydrogels behave similarly to unfilled hydrogels with

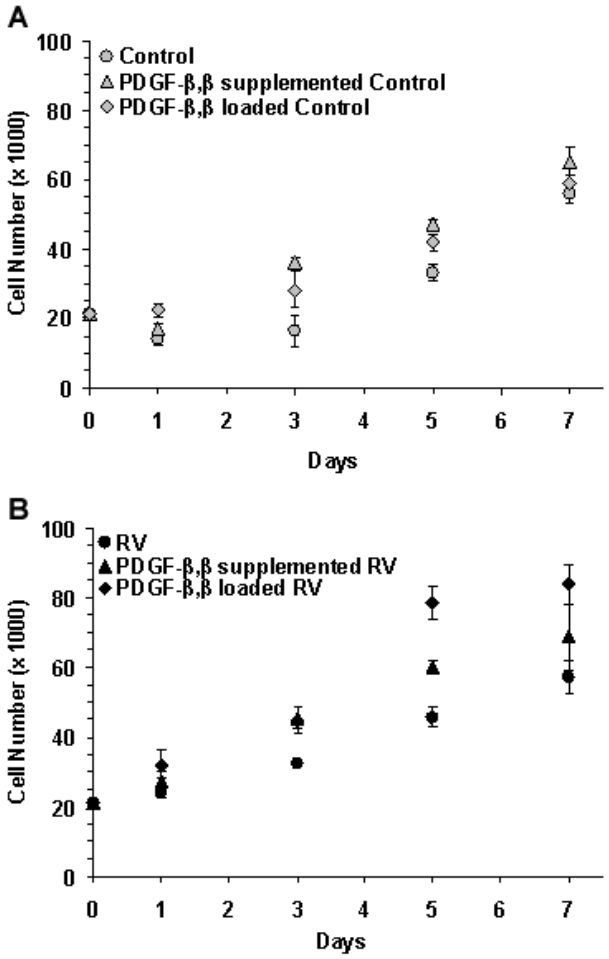

Figure 5. (A) Proliferation of normal human dermal fibroblasts exposed to control hydrogels; (B) proliferation of normal human dermal fibroblasts exposed to release vehicles. Error bars represent standard deviation (n = 4).

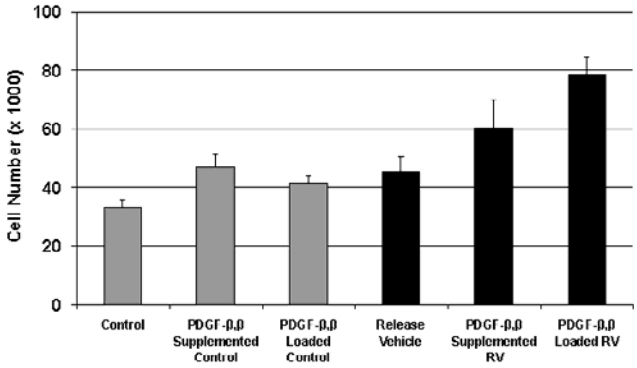

Figure 6. Estimated cell numbers for normal human dermal fibroblasts after 5 days' exposure to controls (grey) and release vehicles (black). Error bars represent standard deviation (n = 4).

the same PVA matrix solid content, they behave more like 20% unfilled hydrogels in terms of their partitioning of TI.

A peak in water uptake was observed in PVA hydrogels with filler added, which may be caused by the hydrophilic nature of the filler (99% hydrolyzed) compared to that of the matrix (83% hydrolyzed). However, this was a transient spike; by 72 hours, the swelling data showed no difference in q, as mentioned above. Taking these results together, it appears that HMW filler is retained in the 15% PVA matrix for at least 5 days and that because of the hydrophilic nature of the added filler, the water uptake profiles are altered during incubation periods, which may affect the free volume available for diffusion. In addition, since partition coefficients were similar in filled hydrogels (15% PVA + 5% filler) and 20% PVA hydrogels, the filler did not appear to influence the affinity of TI for the gel; rather, it contributed to size exclusion.

Fillers are typically added to degradable hydrophilic matrices such as hydroxypropyl methylcellulose (HPMC) and carboxymethylcellulose (CMC) to favorably alter various properties of the vehicle.[21,22] The mechanism of action of water-soluble filler, such as lactose incorporated in HPMC carriers, appears to increase water uptake and facilitate erosion of the carrier.[22] Interestingly, Sako et al[23] found that in vitro drug release only slightly increased in HPMC tablets using either poly(ethylene glycol) or lactose hydrophilic fillers, whereas in vivo release was significantly higher in filled tablets. The difference was attributed to possible effects of mechanical stress on tablet erosion in the gastrointestinal tract.

In the case of CMC hydrogels, studied by Kok et al,[21] the water-insoluble organic compound lignin was used as a filler. The effect of this was to delay drug release, presumably by acting via a filter-like mechanism.[21] In the present study, the hydrophilic filler appears to increase protein release by both altering interactions of the protein with the carrier via size exclusion and changing the water uptake profile, which was characterized by an initial increase in water uptake followed by a steady decrease until equilibrium was reached. Further studies are required to elucidate the mechanisms involved.

This study highlights the point that when HMW compounds are incorporated into hydrogels, the properties of the base hydrogels can be altered dramatically. When different types of biocompatible fillers, such as the PVA fillers with different molecular weights used in this study, are employed, protein drug release can be perturbed without altering the synthesis of the base polymer. This pathway for formulating drug release vehicles is attractive since high flexibility can be obtained without the need to change the attributes of the polymer. In the case of PVA, the attractive properties of being aqueous based and UV crosslinked are not changed, whereas the drug release capacity can be varied over a wide range.

Furthermore, sustained release of PDGF-β,β was induced by incorporation of HMW hydrophilic filler into the hydrogel matrix. For the purpose of this study, a low loading concentration (23.6 ng/gel) was used to determine bioactivity of PDGF-β,β in an in vitro system. Nonetheless, when going into an in vivo model, the amount of growth factor released can be increased by increasing loading concentration, as found in this and other studies.[4,6,9,10] The effectiveness of these sustained release vehicles still needs to be tested in an in vivo wound healing model. To achieve this goal, the loading concentration has to be optimized in order to reach physiological biological activity levels of 1 to 10 ng/day.[9] The hydrogel RV proposed in this study has several practical advantages over existing active wound dressings. It can be fabricated in thin layers that could be applied as a moist wound dressing, and it can be fabricated in several dimensions and trimmed to fit the morphology of the wound. Additionally, since it has a sustained release, it needs to be changed only every few days rather than daily. The shelf life of the preparation was not determined in this study. However, it is clear that use of protease-resistant formulations of PDGF, such as in Regranex, allows significantly longer shelf lives than those for active wound dressings based on cells, such as Apligraf.

Results from the bioactivity assay demonstrated that for the growth factor to have a significant biological effect like proliferation of cells, it should be delivered in a sustained manner. Applying greater amounts in bolus form has less effect probably because of rapid clearance of growth factor from the site or saturation of growth factor receptors. The importance of having sustained release of growth factor (vs bolus application) has been illustrated in an in vivo model for the formation of a mature vasculature by Richardson et al.[13] In addition, the total dose can be minimized when the sustained RV is used.

In conclusion, the release profiles of proteins from PVA hydrogels can be tailored by modifying 3 major parameters: solid content, initial protein loading and incorporation of hydrophilic fillers. With the appropriate combination of these parameters, sustained release of LMW proteins can be obtained from PVA hydrogels. This study provides conclusive evidence that sustained release of bioactive PDGF–β,β from PVA hydrogels occurs for up to 4 days and that PVA hydrogel vehicles

offer an attractive alternative to existing technologies for delivery of protein and peptide active agents.

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